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New insight into the RNA interference response against *cathepsin-L* gene in the pea aphid, *Acyrthosiphon pisum*: Molting or gut phenotypes specifically induced by injection or feeding treatments

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ABSTRACT

RNA interference (RNAi) has been widely and successfully used for gene inactivation in insects, including aphids, where dsRNA administration can be performed either by feeding or microinjection. However, several aspects related to the aphid response to RNAi, as well as the influence of the administration method on tissue response, or the mixed success to observe phenotypes specific to the gene targeted, are still unclear in this insect group. In the present study, we made the first direct comparison of two administration methods (injection or feeding) for delivery of dsRNA targeting the *cathepsin-L* gene in the pea aphid, *Acyrthosiphon pisum*. In order to maximize the possibility of discovering specific phenotypes, the effect of the treatment was analyzed in single individual aphids at the level of five body compartments: the bacteriocytes, the gut, the embryonic chains, the head and the remaining body carcass. Our analysis revealed that gene expression knockdown effect in each single body compartment was dependent on the administration method used, and allowed us to discover new functions for the *cathepsin-L* gene in aphids. Injection of *cathepsin-L* dsRNA was much more effective on carcass and head, inducing body morphology alterations, and suggesting a novel role of this gene in the molting of these insects. Administration by feeding provoked *cathepsin-L* knockdown in the gut and specific gut epithelial cell alteration, therefore allowing a better characterization of tissue specific role of this gene in aphids.

1. Introduction

Aphids (Hemiptera, Aphididae) belong to one of the major families of insect agricultural pests. They are known to cause the most destructive damage on cultivated plants throughout the world, through feeding and as major vectors of plant diseases. As phloem-feeders, some aphids inject a toxic saliva into the affected plants causing a variety of symptoms, including chlorosis, necrosis, wilting, stunting, growth malformation or galls formation (Goggin, 2007), and all of them use the phloem nutrients for their own benefit (Dedryver et al., 2010). The biggest damage caused to crops by aphids is due to the transmission of plant viruses, making aphids responsible for nearly 50% of the transmission of insect-borne viruses in plants (Dedryver et al., 2010).

Over the past decades, control strategies against aphids have been solely based on the use of chemical insecticides directed, in most cases, at neuromuscular targets. Actually, the wide-scale application of such chemicals is becoming increasingly unacceptable as they cause contamination of the environment and food sources, thus constituting a serious health risk to humans (Crinnion, 2009; Komarek et al., 2010). Furthermore, chemical

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insecticides show very low efficiency in preventing crop damage due to aphid-borne viruses (Dedryver et al., 2010). Finally, various aphid species have been found to develop resistance to chemical insecticides (Devonshire, 1989; Furk and Hines, 1993). Consequently, discovering alternative methods for the control of aphids is essential, and a better knowledge of their biology can lead to the development of novel control strategies.

In addition to their importance in agronomy, aphids are also a useful biological model system for studying insect–plant interactions (for a review see (Goggin, 2007)), bacterial-based symbioses (Brinza et al., 2009), insect interactions with parasitoid wasps and predators (Snyder et al., 2006; Dedryver et al., 2010), and phenotypic plasticity (Shigenobu et al., 2010). The recent availability of the pea aphid *Acyrthosiphon pisum* genome (IAGC, 2010) has brought this insect model to a full genomic status, facilitating the development of approaches to discover novel gene functions (Brisson and Stern, 2006; Tagu et al., 2010). Gene inactivation is a key research tool for studying gene function. Although no stable transgenesis techniques have been successfully developed for aphids so far, RNAi-based approaches have already been used to inhibit gene expression in this model where the presence of the siRNA pathway has been previously confirmed by genome analyses (Jaubert-Possamai et al., 2010).

Mutti et al. (2006) were the first to inject siRNA molecules into the abdomen of the pea aphid, obtaining a clear inhibition of the salivary transcript *c002 (ACYPI008617-RA)*. More recently, it was shown that the RNAi transcript inhibition of this same gene affects several aspects of the foraging and feeding behavior of aphids, indicating a crucial role of the protein C002 in aphid feeding on its host-plant, although the mechanism of action of this orphan protein is still totally unknown (Mutti et al., 2008). Jaubert-Possamai et al. (2007) investigated the gene expression knockdown of two genes: *calreticulin* (*ACYPI002622*) and *cathepsin-L* (*ACYPI006974*). They observed, using dsRNA microinjection as a delivery method, up to 40% inhibition of *calreticulin* and 30% of *cathepsin-L* expression. Similar knockdown results were more recently obtained by Guo et al. (2014) on the pea aphid salivary gene *ACYPI39568*. Shakesby et al. (2009) chose aquaporin (*ACYPI006387*), a gut-specific gene, to study the knockdown effects of the dsRNA administration technique by feeding and they observed up to 2-fold inhibition of the target gene. In a multiorganism study, Whyard et al. (2009) showed that the inhibition of *vacATPase* subunit E (*ACYPI009155*), following dsRNA ingestion, could cause a mortality rate of 50% in the pea aphid. More recently, the administration, by feeding, of dsRNA targeting the *hunchback* gene was shown to cause a similar mortality rate in this same aphid species (Mao and Zeng, 2013). Finally, two recent studies have shown the possibility of using plant-mediated RNAi technology to silence genes in the green peach aphid, *Myzus persicae* (Bhatia et al., 2012; Pitino et al., 2013), which is very promising for potential biotechnological applications.

These studies, while revealing new possibilities for the use of RNAi in aphids, also show a relatively high variation in the aphid response to RNAi treatment in terms of efficiency of inactivation, observed mortality rates, or other phenotypic effects. Overall, any conclusions regarding potential differences between the two delivery methods (injection *vs.* ingestion) for RNAi in aphids are very limited as different target genes were inactivated in all these previous studies. Moreover, all these studies have analyzed the gene expression inactivation produced by RNAi on pools of full insect bodies and, to our knowledge, no study has ever measured RNAi-induced gene expression reduction at the level of the tissue/body compartments of individual aphids. It has been shown that, even in organisms where delivering dsRNA causes a systemic response (such as in *Caenorabditis elegans)*, the silencing efficiency is not uniformly distributed in all tissues and it is, for example, difficult to establish an effect in the nervous system (Simmer et al., 2002; Timmons et al., 2001, 2003). The same disparity in RNAi efficiency is observed in *Drosophila melanogaster*, in which wing disk cells appear to be less sensitive than other tissues to RNAi inhibition (Kennerdell and Carthew, 2000). We therefore decided to perform a targeted tissue analysis to study the distribution of the RNAi effects in the pea aphid body. Such an analysis was expected to unravel tissue-specific phenotypes, which otherwise would be impossible to observe in pools of insects.

Cathepsin-L was chosen as a target gene. In many invertebrate groups, cathepsin-L proteases have been identified as major components of the gut digestive enzymes. It has also been demonstrated that they participate in other functions, such as immunological processes and tissue remodeling during insect metamorphosis (Baum et al., 2007; Laycock et al., 1989; Matsumoto et al., 1995; Tryselius and Hultmark, 1997; Hashmi et al., 2002; Wang et al., 2009). These diverse functions across different body compartments in insects make *cathepsin-L* a good candidate for detecting tissue-specific gene knockdown and potential differentiated phenotypes. In aphids, data on the localization and the functional roles of *cathepsin-L* gene are lacking. The few papers published on this subject have shown that the protein is expressed in the gut as an apical membrane-bound enzyme and in the bacteriocytes (Cristofoletti et al., 2003; Deraison et al., 2004), but there is no available information on the expression of *cathepsin-L* gene in other aphid body compartments. The inhibition of cathepsin-L protein activity by a protease inhibitor decreases the development and reproduction rate in *M. persicae* (Cristofoletti et al., 2003), but does not reveal the presence of specific phenotypes related to the treatment. Therefore, the knockdown of *cathepsin-L* gene by injection of dsRNA failed in inducing other phenotypes than some aphid mortality (Jaubert-Possamai et al., 2007).

Our results show, for the first time, that the distribution of the *cathepsin-L* gene knockdown effects vary across the pea aphid tissues and demonstrate how this distribution depends on the dsRNA administration method used. In addition, we show that specific phenotypes are dependent on the inhibition of *cathepsin-L* in different body compartments. Finally, these phenotypes reveal new functions for the *cathepsin-L* gene in the pea aphid.

2. Methods

2.1. Design and synthesis of dsRNA

The selection of the target sequences used in the present study was made using the latest version of the E-RNAi webtool (http://www.dkfz.de/signaling/e-rnai3//) (Arziman et al., 2005; Horn and Boutros, 2010). In this version, the *A. pisum* genome was included following a request from our group to the authors. We were thus able to choose regions of both *cathepsin-L* and EGFP that had no similarities with other transcripts or low-complexity regions in the pea aphid genome. Fragments of *cathepsin-L* and EGFP genes were amplified, by PCR, with cDNA prepared from whole aphid body RNA extracts or the pMP2444 plasmid, respectively. Both primer-end sequences used for dsRNA synthesis included a T7 sequence tail (TAATACGACTCACTATAGGG, Table S1 in Supplementary data) to allow for subsequent dsRNA synthesis. Two picomoles of each PCR product were purified, using the PEG precipitation protocol (Paithankar and Prasad, 1991), and used as templates for dsRNA synthesis. The dsRNAs, synthesized using the MEGAscript T7 kit (Ambion by Life Technologies, Carlsbad, CA, Austin, TX, USA), were then purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified by a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Their quality was

verified using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA).

2.2. Insect rearing

The pea aphids *A. pisum* (Harris) used in this study were obtained from a long established parthenogenetic clone (LL01). The insects were maintained on *Vicia faba* L. (cv. Aguadulce) in ventilated Plexiglas cages, placed in an environmental chamber, at 21 °C, with a photoperiod of 16 h light − 8 h dark. For the microinjection and feeding experiments, the developmental stage of injected aphids was synchronized by collecting newly born nymphs over 24 h. The nymphs were collected either immediately after birth (1st instar), for the feeding experiments, or 5 days later, when they had reached the 3rd instar stage, for the microinjection experiments. For both administration methods, and for the entire duration of the experiments, aphid survival was monitored daily.

2.3. Set-up of dsRNA delivery by microinjection

Synchronized 3rd instar nymphs were injected using an automatic injector apparatus Nanoject II (Drummond Scientific, Broomall, PA, USA) set to slow speed, and with an injection volume not exceeding 46 nl to ensure a low mortality rate in the injected aphids, as previously shown (Jaubert-Possamai et al., 2007). To further optimize this technique with aphid nymphs, we performed several preliminary experiments injecting the insects with sterilized water and changing 3 different parameters: a) the glass capillaries size and type, b) the injection site on the aphid body and c) the method used for aphid immobilization. For these preliminary experiments, 50 aphids were used for each injection group. The aim of this experiment was to reduce accidental mortality linked to the stress induced from manipulation of the aphids.

For the microinjections, we used two kinds of glass capillaries: either pulled-glass capillaries, prepared according to the manufacturer's instructions (3.5inc 3-000-203-G/X micropipettes, Drummond Scientific, Broomall, PA, USA) and using two different "melting" temperatures (65 or 72 °C), or glass needles of 1.0 mm O.D. × 0.78 mm I.D. (Harvard Apparatus, Holliston, MA, USA). The injections were performed at three different sites: a) in the metathorax area between the legs, b) dorsally, or c) laterally, in the middle of the aphid abdomen, between the second and third abdominal segments.

Two different immobilization techniques were used for the injections: aphids were either anesthetized on ice for 5 min, or immobilized with a home-made vacuum-operated insect-holder useful to accurately position the aphids for intra-abdominal injections. In this holder, a yellow tip was placed on the opening of a plastic tube attached to a vacuum pump. An additional side opening could be closed off with tape to regulate the force of the vacuum holding the aphid on the edge of the yellow tip.

Finally, we tested different concentrations of dsRNA (from 2 to 10 μg/μl), injecting 3rd instar nymphs both with dsRNA targeting EGFP (the negative control) or *cathepsin-L* transcripts, in order to ascertain the highest concentration needed to induce effects specific to the target gene and not due simply to the RNAi injection procedure. Higher than 8 μg/μl concentrations killed all injected aphids, even using the negative control EGFP dsRNA.

2.4. Analysis of phenotypes induced by dsRNA microinjection

Having established optimization of the dsRNA microinjection technique, a complete experiment was performed to check the survival rates following *cathepsin-L* dsRNA injection in the pea aphid. Aphids were divided into three treatment groups: injected with *cathepsin-L* dsRNA (75 aphids), or EGFP dsRNA (75 aphids), or water (75 aphids). They were monitored daily over a five-day period. Melanization of the cuticle of the living aphids was always observed at the injection point. For a thorough analysis of *cathepsin-L* dsRNA injection effects, this experimental design ($n = 75$ for each injection group) was repeated and the aphids were followed individually in order to detect a possible induction of external morphological defects or behavior modifications. Five aphids from each injection group were dissected and the morphology of five body compartments evaluated under stereoscopic microscopy at 24, 72 and 120 h after microinjection. For this analysis, the head, the gut, the two ovaries (containing the embryonic chains produced by parthenogenesis in the asexual viviparous aphids used in this study), the bacteriocytes and the remaining body from each aphid were carefully dissected in isoosmotic buffer (pH 7.5, 0.025 M KCl, 0.01 M MgCl2, 0.25 M Sucrose, and 0.035 M Tris−HCl). The tissues were placed in RNA*later*® solution (Ambion by Life Technologies, Carlsbad, CA, USA), stored at −80 °C and then used for qRT-PCR expression analysis. For each tissue coming from single individual aphids, qRT-PCR analysis was performed in triplicate. Each aphid dissected during the experiment was labeled using an individual identifier (Table S2, Supplementary data). All treated aphids and the corresponding dissected tissues, were examined with a MDG-17 stereomicroscope (Leica, Wild Heerbrugg AG, Switzerland).

2.5. Set-up of dsRNA delivery by feeding

For the feeding experiments, aphids were synchronized, as described above, and 1st instar nymphs (aged between 0 and 24 h) were collected and placed on AP3 diet, as described by Febvay et al. (1988). Five days later, after they had all reached the 3rd instar stage, we began the RNAi treatment putting the nymphs onto an AP3 diet, with or without dissolved dsRNA, for a three-day period. At the end of this period, it was necessary to change the artificial diet and all the aphids were put back onto an RNA-free AP3 diet. In order to perform an individual follow-up of the treated aphids, the liquid diet was sealed between two Parafilm layers in a plate 9 mm in diameter and 7.5 mm high (containing 4 μl of artificial diet and allowing for complete nymph development) where one single aphid was placed.

In the same way as for injection experiments, to establish the best conditions for dsRNA administration by feeding, we performed dose response assessments, monitoring the relationship between the dsRNA concentration and survival of aphids. We performed our assessment with doses ranging from 0.9 to 2.6 μg/μl, using both *cathepsin-L* and EGFP dsRNA in order to ascertain the highest concentration needed to induce effects specific to the target gene and not due simply to the dsRNA treatment. Higher than 2 μg/μl concentrations killed all injected aphids, even using the negative control EGFP dsRNA. For these optimization experiments, we used 50 synchronized aphids for each treatment group.

2.6. Analysis of phenotypes induced by feeding on dsRNA

Having established optimization of the dsRNA delivery by feeding, a complete experiment was performed to check the survival rates induced by this treatment in the pea aphid. For this experiment, 336 synchronized 3rd instar nymphs, pre-maintained on dsRNA-free AP3 medium, were separated into three groups: 112 aphids were placed on AP3 with *cathepsin-L* dsRNA at a concentration of 1.8 μg/μl, 112 aphids were placed on AP3 with EGFP dsRNA at a concentration of 1.8 μg/μl and, for the control group, 112 aphids were placed on dsRNA-free AP3. Each aphid was placed on an individual dish containing an artificial diet and monitored daily

over a 5-day period. For a thorough analysis of *cathepsin-L* dsRNA feeding effects, this experimental design ($n = 112$ for each treatment group) was repeated and the aphids were followed individually in order to observe the possible induction of external morphological defects or behavior modifications. Six individuals were collected, from each feeding group, at 24, 72 and 120 h respectively. Aphids were dissected in an iso-osmotic buffer and 5 tissues were isolated as described above. The dissected tissues were then used for qRT-PCR expression analysis. For each tissue coming from single individual aphids, qRT-PCR analysis was performed in triplicate. Each aphid dissected during the experiment was labeled using an individual identifier (Table S2, Supplementary data). All treated aphids, and the corresponding dissected tissues were examined with an MDG-17 stereomicroscope.

2.7. Histology preparations

For histological analyses, the antennae and legs were removed from aphids prior to fixation in Carnoy's solution (absolute ethanol/chloroform/acetic acid − 6/3/1). After 24 h at 4 °C, the fixative solution was replaced with absolute ethanol. Samples were then moved to a 1-butanol solution, at 4 °C, for 24 h. Next, aphids were impregnated and embedded in melted Paraplast (Mc Cormick Scientific LLC, St Louis, USA) and the wax blocks were kept in a dust-free place until sectioning. Tissue sections, 5 μm in thickness, were cut using a LKB Historange microtome (LKB Instruments, Bromma, Sweden) and placed on poly-lysine coated slides, dried overnight in a 37 °C oven, and then kept at 4 °C until staining. Paraffin sections were de-waxed in two baths of methylcyclohexane for 10 min, rinsed in absolute ethanol, and rehydrated through an ethanol gradient to water. Staining was performed using RAL products (RAL reactifs, Martillac, France), according to the following protocol: nuclear staining in Mayer Haemalum for 3 min and washed in water; cytoplasm staining in Eosin solution for 2 min and then washed in water; differentiation in graded ethanol baths, ending with absolute ethanol; collagen staining in alcoholic Saffron for 5 min and an ethanol wash, followed by mounting in Mountex medium (Histolab, Göteborg, Sweden). The tissue preparations were observed under transmitted light, using an Olympus IX81 microscope (Olympus Corporation, Tokyo, Japan) with 10X or 40X lens magnification. Pictures were taken using an Olympus Camedia C-5060 camera (Olympus Corporation, Tokyo, Japan).

2.8. Immunolocalization of cathepsin-L

For the immunohistochemical detection of cathepsin-L, the antennae and legs from aphids were removed prior to fixation of the remaining body in 4% paraformaldehyde in phosphate buffered saline solution (PBS). After one week at 4 °C, the fixative was replaced by several baths of PBS before embedding the aphids in 1.3% agar. Samples were then dehydrated through a series of graded ethanol and moved to a solution of 1-butanol at 4 °C where they were kept for one week. The aphids in agar were then impregnated and embedded with melted Paraplast (Mc Cormick Scientific LLC, St Louis, MO, USA). The wax blocks were kept in a dust-free place until sectioning. Tissue sections, 5 μm thick, were cut using an LKB Historange microtome (LKB Instruments, Bromma, Sweden) and the sections were placed on poly-lysine coated slides, dried overnight in a 37 °C oven, and then maintained at 4 °C until immunostaining. Paraffin sections were de-waxed in 2 baths of methylcyclohexane for 10 min, rinsed in absolute ethanol, and rehydrated, through an ethanol gradient, to PBS. Slides were incubated with 1% bovine serum albumin (BSA) in PBS for 30 min prior to a primary incubation overnight, at 4 °C, with a monoclonal anti-insect cathepsin-L antibody (mouse antibody raised against *Spodoptera frugiperda* cathepsin-L, clone 193702, R&D Systems, Minneapolis, MN, USA), diluted to 1:200 in BSA 0.1%. BSA 0.1% was used as a negative control. After primary antiserum incubation, sections were washed with PBS containing 0.2% Tween-20. Primary antibodies were detected with a fluorescent goat anti-mouse IgG, coupled to Alexa Fluor® 568 (Molecular Probes by Life Technologies Ltd, Carlsbad, CA, USA). This secondary antibody was applied for 1 h, at room temperature, diluted to 1:500 in BSA 0.1% in PBS. From this step onwards, all manipulations were carried out in the dark. Next, sections were washed with PBS-Tween, rinsed with PBS and with several baths of tap water. The sections were left to dry and then mounted, using PermaFluor™ Aqueous Mounting Medium (ThermoFisher Scientific, Cheshire, UK) together with 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining (3 μg per ml of medium). Sections were observed under an epi-fluorescence IX81 Olympus microscope, using specific emission filters: HQ610/75 for a red signal (antibody staining), D470/40 for a blue signal (DAPI) and HQ535/50 for a green signal (non-specific autofluorescence from tissues). Microphotographs were captured and modified using an F-View II camera coupled with the Cell F Software (Olympus SIS GmbH, Münster, Germany).

2.9. Real-time quantitative RT-PCR

Total RNA was extracted from the dissected tissues using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Twenty ng of the total RNA extracted were treated with RQ1 RNase-Free DNase I (Promega Corporation, Madison, WI, USA), and 10 ng of it were reverse-transcribed with the Sensiscript® RT kit (Qiagen, Hilden, Germany), using Oligo(dT) primers (Invitrogen by Life Technologies, Carlsbad, CA, USA) in order to obtain first-strand cDNA. The quality of the cDNA synthesized was examined by PCR, using 1 μl of the cDNA produced, as a template, and gene specific primers for actin in a concentration of 2 μM per reaction. As a negative control, the remainder of the DNase treated RNA (10 ng) was examined by PCR using the same conditions.

Real-time RT-PCR reactions were performed on a LightCycler® 480 Real-Time PCR System (Roche, Penzberg, Germany) using 1:2.5 diluted cDNAs and a LightCycler® 480 SYBR Green I Master Mix (Roche, Penzberg, Germany) according to the manufacturer's instructions. For each gene, triplicate assays were performed. The specific primer pair *cathepsin*_F/*cathepsin*_R was used to amplify the *cathepsin-L* gene. In order to choose the best normalization gene for our study, we compared the gene expression levels of 5 candidate genes: *actin* (*ACYPI000064*), *cyclophilin* (*ACYPI003541*), *gadph* (*ACYPI008372*), *rpl7* (*ACYPI010200*) and *rpl32* (*ACYPI000074*). The gene expression variation in all the experimental conditions tested in this work for these five genes were tested with the BestKeeper software tool (Pfaffl et al., 2004). Only the *rpl32* gene was retained as a normalization gene as it met the criteria imposed by the BestKeeper analysis: standard deviation ≤ 1 CP (Crossing Point) between the different tested conditions for each experimental point (data not shown). All the primers used in this study are listed in Table S1 (Supplementary data).

2.10. Statistical analyses

For mortality scoring, all data were analyzed by non-parametric (Kaplan Meier) and parametric survival analysis using the JMP® software (SAS institute, Cary NC USA). As a result of the experimental design employed (insects always caged individually), each individual aphid score was a true independent measure, with n ranging between 50 and 112 according to the experiment and the group. Individuals still alive at the end of experiment were labeled as censored on the last observation day. The effect of the treatment

on survival was tested using the Log-rank statistics (Chi2 at 1 df, performed on three or two groups, either with both controls or with the EGFP control alone).

For the real-time quantitative RT-PCR experiments, the gene expression levels of all target genes were calculated and normalized using the REST software tool (Pfaffl et al., 2002). The expression levels (log2) of the *cathepsin-L* gene were normalized relative to those of the *rpL32* gene. The relative expression ratio R (and the associated standard error) of *cathepsin-L* mRNA in the different tissues of individuals treated with *cathepsin-L* dsRNA (injection or feeding) was calculated using as a reference the mean of the *cathepsin-L* transcript expression levels of the aphid control group (water or AP3 diet, and EGFP). More precisely, this ratio was calculated taking into account the real-time PCR efficiency of each gene (E) and the crossing point difference (ΔCP) of a test condition, as compared to the reference condition, and expressed in comparison to the normalization gene (*rpl32*) using the following model (Pfaffl, 2001):

$$R = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(\text{control}-\text{sample})}}{(E_{\text{reference}})^{\Delta \text{CP}_{\text{reference}}(\text{control}-\text{sample})}}$$

Statistically significant *cathepsin-L* knockdowns, calculated by the REST analysis, are shown in bold in Tables S3 and S4 (Supplementary data).

3. Results

3.1. Expression profiles of cathepsin-L mRNA in aphid body compartments

Before checking the inactivation of *cathepsin-L* gene expression, RT-PCR experiments were performed on total RNA extracted from different aphid body tissues. Cathepsin-L encoding transcripts were detected at similar levels in all of the five body compartments from *A. pisum* tested here, when compared to the ribosomal protein coding gene *rpl32 (ACYPI000074)* (Fig. S1, Supplementary data).

3.2. Optimization of the dsRNA administration by microinjection

Microinjection in small insects, such as aphids, is a critical point in RNAi approaches. To improve the existing microinjection protocols, that are known to induce high mortality in aphids even within control groups (29% mortality, observed by Jaubert-Possamai et al. (2007), after injecting 3rd instar nymphs with 46 nl of water), we performed several experiments in order to define the best injection procedure, as well the optimal dsRNA concentration.

In our experiments, microinjections using the 1.0 mm O.D. × 0.78 mm I.D. capillaries resulted in much lower mortality rates when compared to other capillaries. Immobilization using a vacuum-holder, applying a technique similar to that used by Mutti et al. (2006), was more effective than the partial anesthetization of the insects on ice, as previously used by Jaubert-Possamai et al. (2007). Furthermore, we found that injections performed laterally in the aphid body and directed towards the abdomen, between the 2nd and the 3rd abdominal segment on the height of the leg, resulted in the lowest out-flow of body fluid. This injection site resulted in lower mortality rates than injections performed dorsally, as in Jaubert-Possamai et al. (2007), or between legs, as in Mutti et al. (2006). Taking into account all these different parameters, we were able to reduce aphid mortality from 30% to 5% in the water control group.

Finally, our data showed that the dsRNA concentration should not exceed 8 μg/μl (in a volume of 46 nl) as, above that value, even negative control EGFP dsRNA caused significant mortality in the aphids. The observed high dose toxicity of dsRNA has been previously, and in other model systems, attributed to a non-specific saturation of RNAi machinery causing a consequent alteration of the microRNA pathway (Grimm et al., 2006). Following these results, all the microinjection experiments in this study were performed with a dsRNA concentration of 7 μg/μl.

3.3. Survival rates and phenotypes after dsRNA administration by microinjection

In order to characterize precisely the effects of *cathepsin-L* gene down-regulation by RNAi treatment in individual aphids, a total of 225 insects (75 for each injection group: water, EGFP or *cathepsin-L* dsRNA) were followed daily for five days to check their survival and the occurrence of body morphology phenotypes. With the exception of the first day, the mortality of aphids injected with *cathepsin-L* dsRNA was always significantly higher than that of aphids from other injection groups (Table 1, Injection treatments, Experiment 1). Survival analysis, with a Weibull fit adjustment (Fig. 1), revealed that this treatment had a significant effect on both controls, with a decrease in the mean survival time of the first 20% of the population from 141 ± 12 h (EGFP control) to 63 ± 6 h (*cathepsin-L*). This corresponds to a significant increase in the final cumulated mortality from 19% for EGFP to 31% for *cathepsin-L* treated aphids ($p = 0.082$ for a $\chi 2$ test of the whole parametric model, and $p = 0.069$ in comparison to the water control). This experiment allowed us to detect a significantly higher number of behavior alterations and morphological defects in treated aphids than in the controls (Table 1, Injection treatment, Experiment 1).

Starting from the sixth day after microinjection, aphids were monitored every 48 h for a supplementary period of 10 days. All injected aphids surviving the treatment gave birth to correctly developed nymphs at a morphological level.

The same experiment was repeated: 75 aphids for each injection group were followed during a period of 5 days. This allowed us to detect that, 24 h after the injections, an approximately 20% of the aphids injected with *cathepsin-L* dsRNA showed morphological defects, which was significantly different from the control groups ($p < 0.01$, Table 1, Injection treatments, Experiment 2). The body of these aphids was deformed, resulting in a non-uniform insect shape (example in Fig. 2). A bigger proportion (50%) of the aphids injected with *cathepsin-L* dsRNA (and all the aphids with morphological defects) showed reduced mobility and they were easily detached from plant leaves. Five aphids from each treatment group were dissected and the stereoscopical examination of the body compartments showed that no visible alteration was affecting their bodies. In order to examine whether the phenotype observed was related to *cathepsin-L* gene inhibition, we used aphids presenting an altered morphology for the tissue gene expression analysis by qRT-PCR (for example aphids labeled ICD and ICE in Fig. 3, and Table S2 in Supplementary data), as well as the aphids that did not show visible phenotypic effects.

3.4. Tissue gene expression analysis after dsRNA administration by microinjection

For all time points (24, 72 and 120 h), *cathepsin-L* transcript levels in five selected body compartments (bacteriocytes, embryonic chains, gut, head and the remaining body), dissected from each of the five aphids that were examined with a stereoscopic microscope following microinjection, were measured by qRT-PCR. The aphids' developmental stages were synchronized, excluding any

Table 1
Overview of phenotypes observed after treatment with *cathepsin-L* dsRNA by injection or feeding in *A. pisum* (cumulative effects over 5 days of treatment).

Injection treatments	Control (water)	EGFP dsRNA	*cathepsin-L* dsRNA
Experiment 1			
Mortality (75 aphids from each treatment group)	7/75	12/75 (p[a] = 0.33)	23/75 ($p = 1.9\ 10^{-3}$)
Behavior alteration (75 aphids from each treatment group)	2/75	1/75 ($p = 1$)	38/75 ($p = 4.5\ 10^{-12}$)
Cuticle/body shape alteration (75 aphids from each treatment group)	1/75	1/75 ($p = 1$)	14/75 ($p = 5.5\ 10^{-4}$)
Experiment 2			
Behavior alteration (75 aphids from each treatment group)	0/75	0/75 ($p = 1$)	37/75 ($p = 3.7\ 10^{-14}$)
Cuticle/body shape alteration (75 aphids from each treatment group)	0/75	0/75 ($p = 1$)	15/75 ($p = 2.8\ 10^{-5}$)
Statistically significant *cathepsin-L* knockdown in at least 1 body compartment (15 aphids from the *cathepsin-L* group)	–	–	12/15[b]
Statistically significant *cathepsin-L* knockdown in all the analyzed body compartments (5 tissues × 15 aphids = 75 tissues)	–	–	26/75[c]
Feeding treatments	**Control (AP3)**	**EGFP dsRNA**	***cathepsin-L* dsRNA**
Experiment 1			
Mortality (112 aphids for each treatment group)	0/112	19/112 ($p = 1.7\ 10^{-6}$)	43/112 ($p = 1.5\ 10^{-15}$)
Behavior and/or body morphology alteration (112 aphids for each treatment group)	1/112	2/112 ($p = 1$)	1/112 ($p = 1$)
Experiment 2			
Behavior and/or body morphology alteration (112 aphids for each treatment group)	1/112	0/112 ($p = 1$)	2/112 ($p = 1$)
External gut alteration observed by stereoscopical analysis (18 aphids for each treatment group)	0/18	2/18 ($p = 0.49$)	7/18 ($p = 7.6\ 10^{-3}$)
Statistically significant *cathepsin-L* knockdown in at least 1 body compartment (18 aphids from the *cathepsin-L* group)	–	–	8/18[b]
Statistically significant *cathepsin-L* knockdown in all the analyzed body compartments (5 tissues × 18 aphids = 90 tissues)	–	–	9/90[c]
Experiment 3			
Gut histological alteration (18 aphids from each treatment group)	0/18	2/18 ($p = 0.49$)	18/18 ($p = 2.2\ 10^{-10}$)

[a] The p-values are calculated using Fisher's exact test to compare proportions between a treatment (EGFP dsRNA or *cathepsin-L* dsRNA) and the control (water for injection or AP3 for feeding).
[b] Comparison of significant *cathepsin-L* gene knockdown in at least 1 body compartment between injection and feeding: $p = 0.072$.
[c] Comparison of significant *cathepsin-L* gene knockdown in all the analyzed body compartments between injection and feeding: $p = 2.1\ 10^{-4}$.

possible gene expression variation due to this parameter. Nevertheless, the expression levels of *cathepsin-L* in the same aphid tissue, showed significant variation between individuals. To take into account this variation (and to distinguish it from the effect of the injection), the expression of the *cathepsin-L* gene was normalized relative to the gene *rpl32* and by using, as a reference, the mean of the *cathepsin-L* mRNA levels in each body compartment of the control groups (water and EGFP). Statistically significant *cathepsin-L* knockdowns calculated by the REST analysis, are shown in bold in Table S3 (Supplementary data) and summarized in Fig. 3.

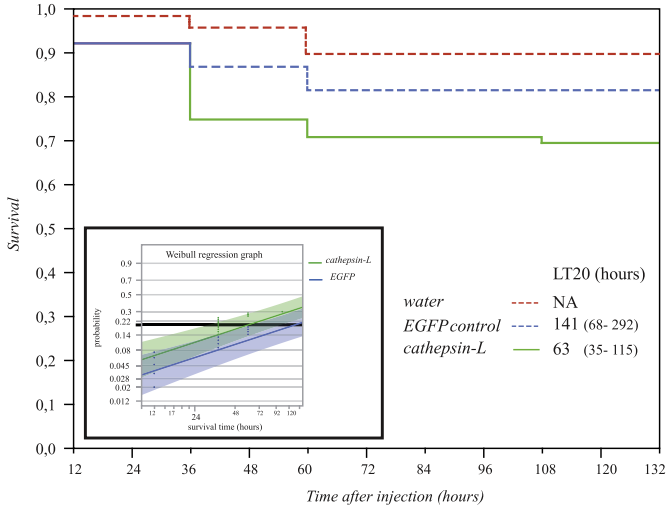

Fig. 1. Survival curves of aphids injected with dsRNA. The different lines represent the survival curves for the different groups of injected aphids: aphids injected with *cathepsin-L* dsRNA (solid green line), aphids injected with EGFP dsRNA (dotted blue line) and aphids injected with sterilized water (dotted red line). The insert shows a Weibull-fit graph of the data, allowing for a parametric estimation of the LT20s in the two dsRNA treated groups (mean times at which 20% of the population has died; numbers in brackets show 5% confidence intervals).

Twenty-four hours after the injections, we observed a decrease in *cathepsin-L* transcripts in 3 out of the five individuals injected with *cathepsin-L* dsRNA, compared to the controls (Fig. 3). The strongest inhibition of the target gene was observed in the carcasses (up to 2.5-fold compared to the controls) and in the embryonic chains (up to 1.9) (Table S3, Supplementary data). Interestingly, the two aphids with the highest level of *cathepsin-L* gene expression down-regulation were ICD and ICE individuals, which presented the morphological defects shown in Fig. 2.

Seventy-two hours after the injections, all aphids injected with *cathepsin-L* dsRNA showed lower *cathepsin-L* transcript levels compared to the control group (Fig. 3). The strongest inhibition of the

Fig. 2. Phenotypes induced by microinjection of *cathepsin-L* dsRNA. An aphid, injected with *cathepsin-L* dsRNA and showing morphological external defects (on the right), is compared with a healthy injected aphid (on the left). The arrows indicate the regions in the aphid body where the defects are the most evident. S: sick aphid, m: melanization point. Scale bar is 1 mm.

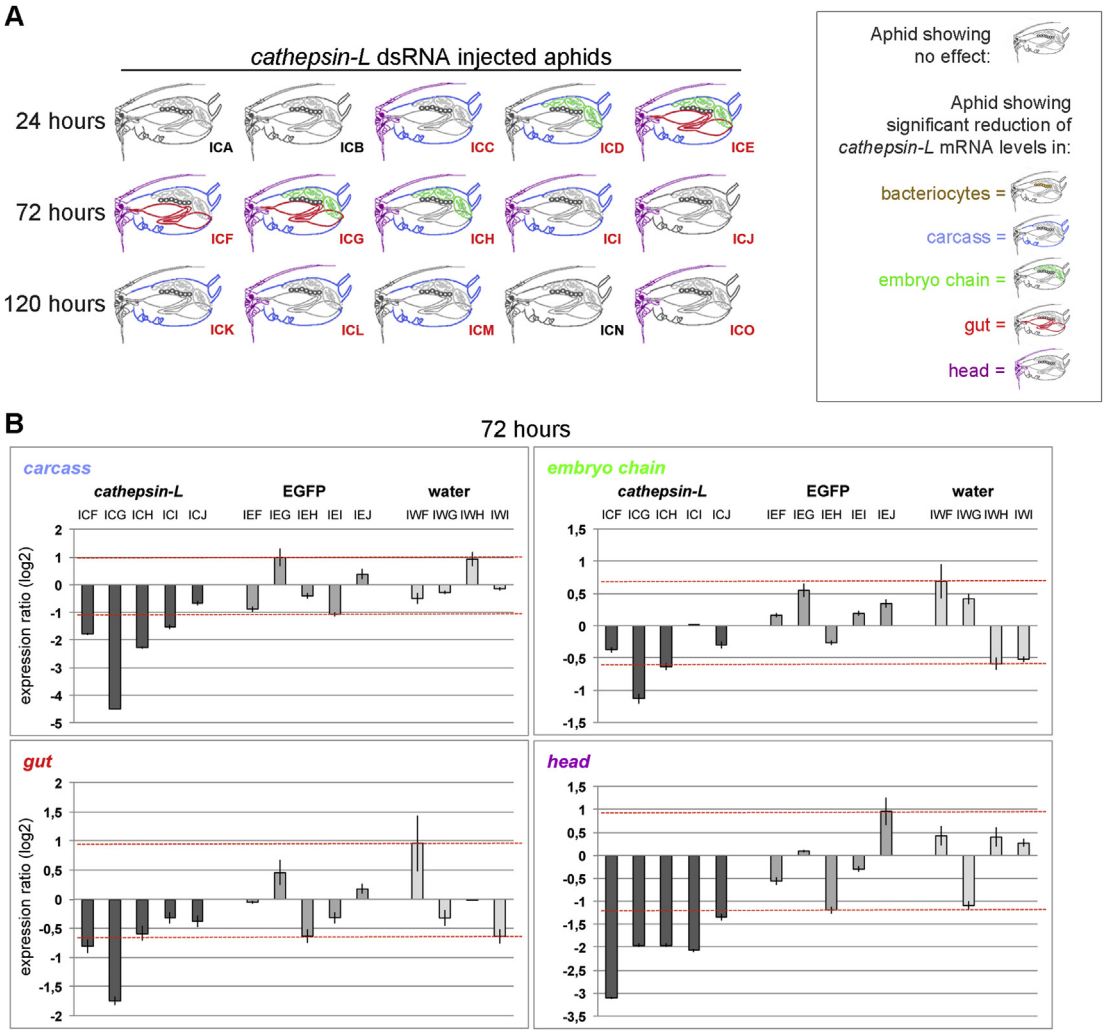

Fig. 3. *cathepsin-L* gene knockdown in the microinjection experiment. (A) This diagram summarizes the results of the qRT-PCR experiments performed to analyze *cathepsin-L* mRNA knockdown following dsRNA treatment by microinjection in the pea aphid. Five different body compartments were analyzed separately in five individual aphids 24, 72 and 120 h after the beginning of the treatment. Significant inhibition in each tissue is represented by a different color, while the non-inhibited tissues are represented by different shades of grey. Each individual aphid is labeled as indicated in Table S2 (Supplementary data) and the labels of aphids showing a statistically significant reduction of *cathepsin-L* mRNA level in at least one tissue are in red. A detailed report of all the results obtained from this dataset is shown in Table S3 (Supplementary data). (B) Expression of *cathepsin*-L mRNA after normalization with the *rpl32 gene* in representative tissues (embryonic chains, head, carcass and gut) 72 h after the microinjections. Quantitative real-time PCR experiments and data analysis were performed as described in the Methods section. The results obtained for each tissue from aphids injected with the *cathepsin-L* dsRNA, EGFP dsRNA and water, are shown here from left to right, respectively. Negative expression ratios indicate an under-expression and positive ratios indicate an over-expression of the gene. The dashed red lines indicate the boundaries of *cathepsin-L* mRNA level variation in the control groups (water and EGFP).

target gene expression was observed in the carcasses (up to 4.5-fold, for four out of the five aphids analyzed here) and the heads (up to 3.1-fold, for all five aphids) (Fig. 3B, and Table S3 in Supplementary data). A significant inhibition of *cathepsin-L* expression was also observed in the gut and in the embryonic chains in two out of the five aphids analyzed (Fig. 3, and Table S3 in Supplementary data). All the aphids with morphological defects showed reduced *cathepsin-L* mRNA levels (ICF, ICG and ICI individuals).

One hundred and twenty hours after the injections, a significant inhibition of the target gene mRNA level was still observed in the carcasses (up to 2.9-fold lower than the controls for 3 out of five individuals) and the heads (up to 1.8-fold lower than the controls for two out of five individuals) of aphids injected with *cathepsin-L* dsRNA (Fig. 3, and Table S3 in Supplementary data). No significant reduction in the *cathepsin-L* expression levels among the different injection groups was observed in the bacteriocytes or the embryonic chains. On the other hand, an induction of the *cathepsin-L* expression levels was observed in the guts of aphids injected with *cathepsin-L* (Table S3, Supplementary data). This intriguing result is similar to that found by Jaubert-Possamai et al. (2007) in pools analysis following *cathepsin-L* dsRNA injection in the same aphid species.

In summary, over five days of treatment, a statistically significant knockdown of *cathepsin-L* gene expression was observed in 12 out of the 15 aphids analyzed here (Table 1, Injection treatment, Experiment 2). The knockdown was the highest at 72 h and variable depending from single individual aphids and tissues. The most sensitive tissue to the *cathepsin-L* dsRNA injection was the carcass. It is to mention that our sampling strategy (selection of surviving insects for the qRT-PCR analyses) may have created an underestimation of the number of insects showing a statistically significant gene expression knockdown: it is possible that aphids having the highest *cathepsin-L* gene inhibition were among the ones that died and could not be examined.

3.5. Optimization of the dsRNA administration by ingestion

The dose response assessments performed in our study showed that the highest amount of dsRNA that could be administered to 3rd instar aphid nymphs by feeding, whilst maintaining a relatively low mortality, was ranging between 1.8 μg/μl and 1.9 μg/μl over a 3-day period. At higher concentrations, the treated aphids died independently from the administered dsRNA. Hence, in our feeding experiments, in order to select a dsRNA concentration able to induce gene specific effects, we decided to perform our phenotypic and tissue analysis in aphids fed on artificial diets containing 1.8 μg/μl dsRNA.

3.6. Survival rates and phenotypes after dsRNA administration by ingestion

After the administration of dsRNA by feeding, we monitored insects daily to evaluate survival rates, over a five-day period, for 336 aphids (112 for each treatment) fed on *cathepsin-L* dsRNA, EGFP dsRNA or dsRNA-free AP3 artificial diets. No mortality was observed during the first 48 h (Fig. 4). After 96 h, a mortality rate of approximately 14% and 36% was observed in the aphids fed on EGFP dsRNA and on *cathepsin-L* dsRNA, respectively. The final increase in mortality for the ingestion assay (relative to the AP3 control group) was between 19% (EGFP control, as for the injection group) and 46% (*cathepsin-L*, higher than the injection group). In the feeding treatment, the effect of *cathepsin-L* RNAi ingestion was very significant ($p < 0.0001$ for a $\chi 2$ test of the whole model, after a Weibull-fit survival analysis; Fig. 4). Conversely to the microinjection treatment, aphids did not show modification in their behavior or their body shape after *cathepsin-L* dsRNA feeding treatment (Table 1, Feeding treatments, Experiment 1).

The same experiment was repeated: 112 aphids for each feeding group were followed during a period of 5 days. Again, no behavioral changes or morphological alterations were observed for the aphids fed on *cathepsin-L* or EGFP dsRNAs, compared to those fed on the control diet (Table 1, Feeding treatments, Experiment 2).

During the experiment, six aphids from each experimental group were dissected and evaluated, using stereoscopic microscopy, 24, 72 and 120 h after the administration of dsRNA. The dissected tissues were then analyzed with the qRT-PCR technique (six aphids from the *cathepsin-L* group, and four aphids from the control groups). The stereoscopic analysis revealed several differences in the morphology of the stomach among the aphids fed on the different diets. While normally the stomach of aphids appeared to be dense under the microscope, with a layer of epithelial cells surrounding it, in several dsRNA treated aphids it was transparent and occasionally filled with granules and granular formations (Fig. S2, Supplementary data). We observed this phenotype significantly more frequently (seven out of the 18 aphids analyzed at the end of the observations, $p < 0.01$) in the aphids fed on *cathepsin-L* dsRNA than in aphids of the control groups (Table 1, Feeding treatments, Experiment 2).

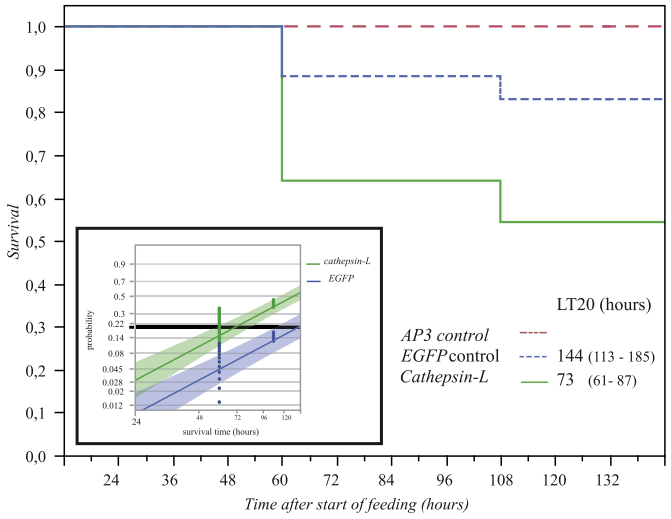

Fig. 4. Survival curves of aphids fed on artificial diets supplemented with dsRNA. The different lines represent the survival curves for the three groups of aphids: aphids fed on *cathepsin-L* dsRNA/AP3 (solid green line), aphids fed on EGFP dsRNA/AP3 (dotted blue line) and aphids fed on AP3 (dotted red line). The insert shows a Weibull-fit graph of the data, allowing for a parametric estimation of the LT20s in the two dsRNA treated groups (mean times at which 20% of the population has died; numbers in brackets show 5% confidence intervals).

Following these observations, we repeated the feeding experiment on a smaller group of aphids (50 aphids for each feeding group) and we evaluated the morphology of the aphid gut cells at the histological level using hematoxylin/eosine staining using six aphids from each diet and for each time point. This led to the discovery that, in all aphids fed on *cathepsin-L* dsRNA, the integral structure of the gut was deteriorating from 24 to 120 h after the administration, as indicated by vacuole formation (a sign of cell necrosis) and destruction of the epithelial cell membranes from 24 h onwards (Fig. 5E,F). These vacuoles were observed in the intestines of all *cathepsin-L* dsRNA treated aphids, but rarely in the stomachs, where they appeared only from 72 h after the beginning of the treatment. The microscopy analysis of these aphids also revealed an alteration in their stomachs, where we frequently observed lyzed epithelial cells (Fig. 5E). Conversely, histological alterations were not found in either the intestines or the stomachs of aphids fed on EGFP dsRNA (Fig. 5A–D and Table 1, Feeding treatments, Experiment 3). Vacuole structures were rare in the stomachs of aphids fed on EGFP dsRNA or on dsRNA-free AP3.

3.7. Tissue expression analysis of aphids after dsRNA administration by ingestion

For each of the time points described above (24, 72 and 120 h), the *cathepsin-L* transcript levels in five selected body compartments (bacteriocytes, carcass, embryonic chains, gut and head) dissected from aphids analyzed by stereoscopical examination for the three treatment groups, were measured by qRT-PCR. Six aphids were examined from the *cathepsin-L* group and four aphids were examined from both control groups (EGFP and AP3 diet). Again, a variation in the expression levels of the *cathepsin-L* transcripts was observed among the aphid tissues of the different feeding groups, and also among tissue samples from aphids within the same feeding group. In order to take this variability into account, as regards the injection study, the expression level of the *cathepsin-L* gene was normalized relative to the gene *rpl32* using, as a reference, the mean of the *cathepsin-L* mRNA levels in each body compartment of the individuals from the control groups (EGFP dsRNA and dsRNA-free AP3). Statistically significant *cathepsin-L* knockdowns calculated by the REST analysis, are shown in bold in Table S4 (Supplementary data) and summarized in Fig. 6.

Twenty-four hours after the administration of dsRNA, we observed that the aphids fed on dsRNA had higher *cathepsin-L* expression levels compared to the aphids fed on control diets, with induction varying among individuals and tissues (Table S4, Supplementary data). In order to exclude the possibility that the *cathepsin-L* induction was an artifact produced by the assay conditions, we repeated the feeding experiment and performed new independent qRT-PCR assays. In these experiments, the GAPDH-encoding gene was used to normalize the data (data not shown).

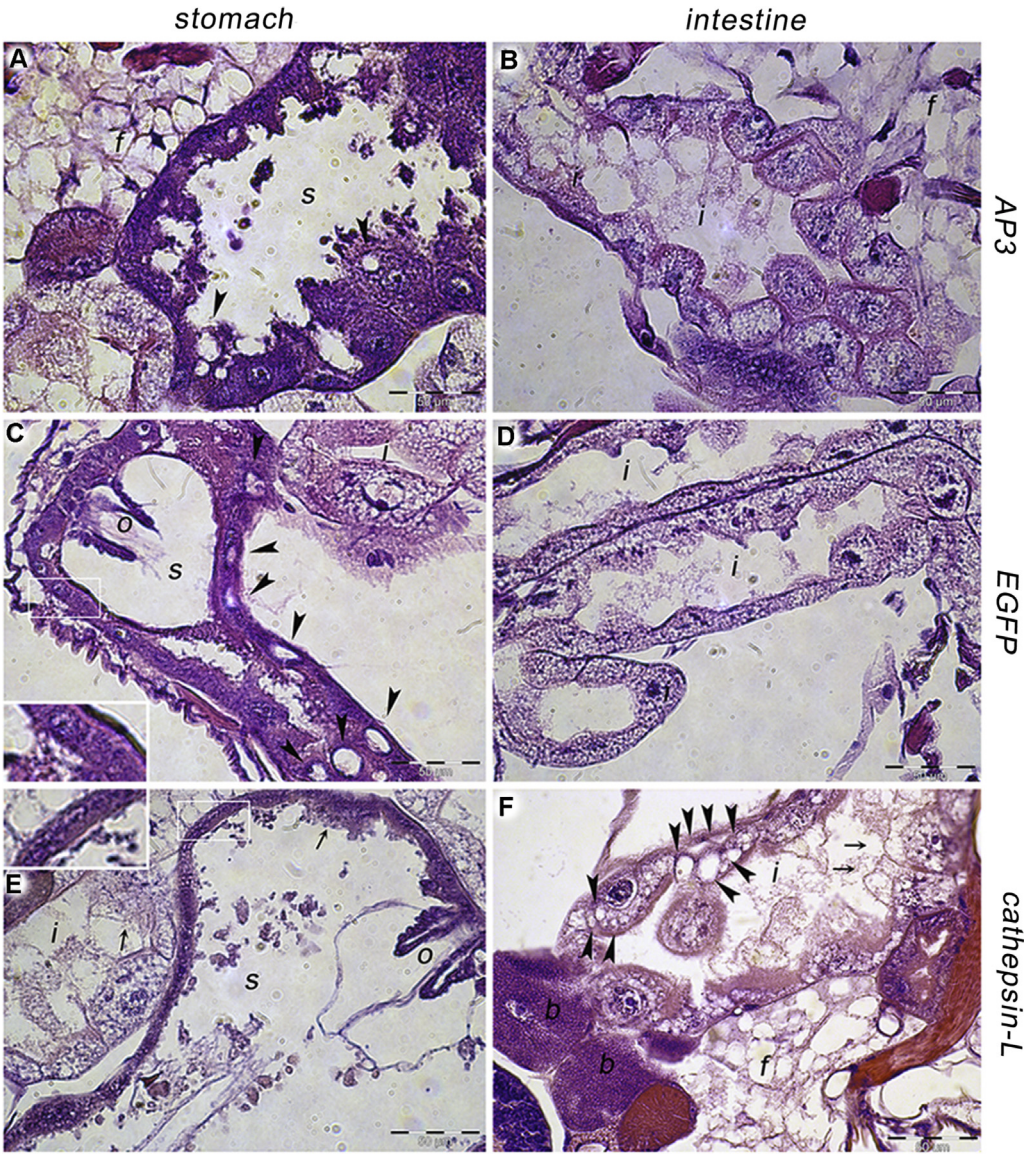

Fig. 5. Hematoxylin-eosine staining of guts from aphids fed on dsRNA-supplemented diets. The hematoxylin-eosine stainings were performed, as described in the Methods section, on aphids from each feeding group 120 h after the beginning of the RNAi treatment. Sections are of the stomach (A, C and E) or of the intestine (B, D, F) of aphids fed on dsRNA-free AP3 diet, EGFP dsRNA supplemented AP3 diet or *cathepsin-L* dsRNA supplemented diet. The small frame in C represents a 2× magnification view showing details of the epithelial cell organization. The small frame in E shows the change in epithelial cell organization in the stomachs from aphids fed on *cathepsin-L* dsRNA supplemented AP3 diet. S: stomach, f: fat body, i: intestine, o: esophagus, b: bacteriocytes. Large arrowheads show vacuoles, small arrows show degenerated cells with lyzed membranes. All scale bars are 50 μm.

This allowed us to confirm the *cathepsin-L* mRNA induction observed 24 h after the administration of dsRNA.

Seventy-two hours after the beginning of the experiment, the *cathepsin-L* expression levels in the gut of four out of the six aphids analyzed (FCI, FCJ, FCK and FCL individuals) and the head of one out of the six aphids analyzed (FCK individual), and fed on *cathepsin-L* dsRNA, were reduced compared to the controls (Fig. 6, and Table S4 in Supplementary data). All these aphids belonged to the group showing altered gut morphology upon stereoscopical observation.

One hundred and twenty hours after the beginning of the treatment a reduction in *cathepsin-L* expression levels was observed only in the gut of one out of the six individuals analyzed and the bacteriocytes of three out of the six individuals analyzed (Fig. 6, and Table S4 in Supplementary data).

In summary, over five days of treatment, a statistically significant knockdown of *cathepsin-L* gene expression was observed in eight out of the 18 aphids analyzed here (Table 1, Feeding treatments, Experiment 2). The most sensitive tissue to the *cathepsin-L* dsRNA treatment by feeding was the gut. As for injection experiments, our sampling strategy (selection of surviving aphids for the qRT-PCR analyses) may have created an underestimation of the number of insects showing a statistically significant *cathepsin-L* knockdown.

4. Discussion

One objective of the present work was to investigate the differences between two dsRNA administration methods (injection *vs.* ingestion), with respect to specific tissue and individual responses to RNAi treatment in aphids. The gene target in our study was *cathepsin-L*, a gene initially described as being mainly expressed in the gut and the bacteriocytes in aphids (Deraison et al., 2004).

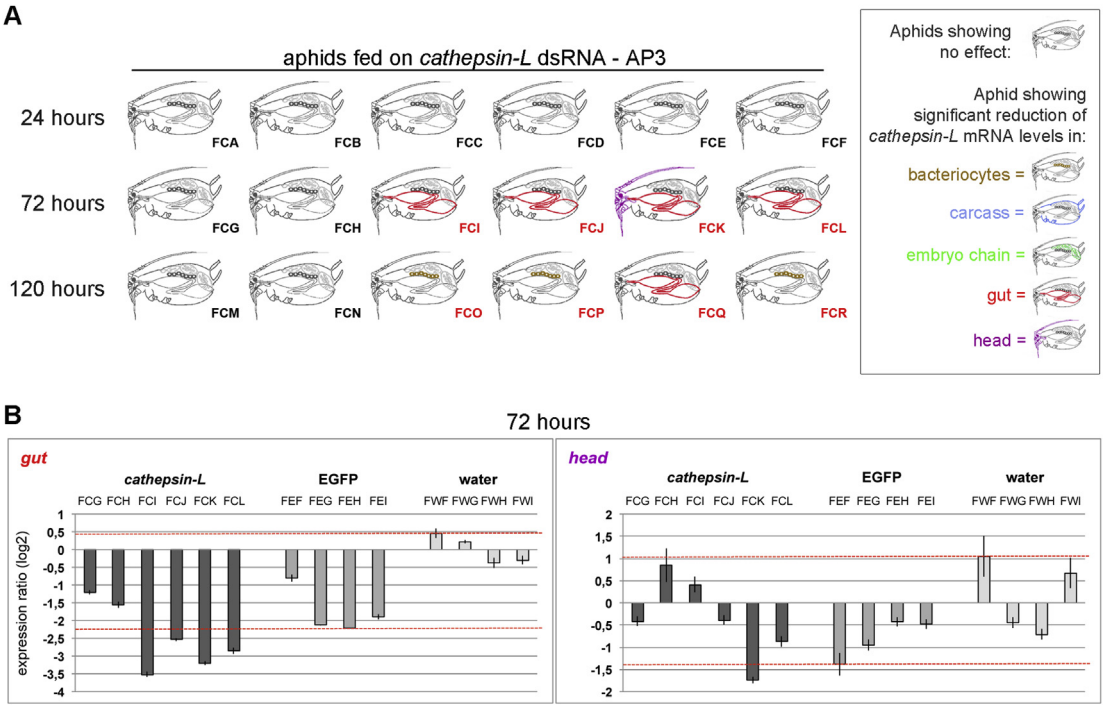

Fig. 6. *cathepsin*-L gene knockdown in the feeding experiment. (A) This diagram summarizes the results of the qRT-PCR experiments performed to analyze *cathepsin-L* mRNA knockdown following RNAi treatment by feeding. Five different body compartments were analyzed separately, in six individual aphids, 24, 72 and 120 h after the beginning of the treatment. Significant inhibition in each tissue is represented by a different color, while the non-inhibited tissues are represented by different shades of grey. Each individual aphid is labeled, as indicated in Table S2 (Supplementary data) and the labels of aphids showing a statistically significant reduction of *cathepsin-L* mRNA level in at least one tissue are in red. A detailed report of all the results obtained from this dataset is shown in Table S4 (Supplementary data). (B) Expression of *cathepsin*-L mRNA after normalization with the *rpl32 gene* in representative tissues (the gut and the head) 72 h after the beginning of the treatment. Quantitative real-time PCR experiments and data analysis were performed as described in the Methods section. The results obtained for each tissue from aphids fed on *cathepsin-L* dsRNA supplemented AP3 diet, EGFP dsRNA supplemented AP3 diet and dsRNA-free AP3 diet, are shown here from left to right, respectively. Negative expression ratios indicate an under-expression and positive ratios indicate an over-expression of the gene. The dashed red lines indicate the boundaries of cathepsin-L mRNA level variation in the control groups (dsRNA-free AP3 and EGFP dsRNA AP3 diets).

However, we found this gene to be expressed also in other parts of the aphid body (Fig. S1), and this allowed us to monitor its inhibition in all the different body compartments analyzed in this work. We have shown, for the first time in aphids, that the two administration methods for delivering dsRNA induce different phenotypic effects in *A. pisum* when used for the inactivation of the same target gene (see Table 1 for an overview of different phenotypes observed with injection or feeding). First, the body compartments targeted by the gene knockdown are specific to the administration method used. Indeed, microinjection of dsRNA targeting the *cathepsin-L* gene in aphids resulted in significant gene knockdown in the carcass, the head, the gut and the embryonic chains. Administration by feeding resulted in a clear gene knockdown in the aphid's gut, together with mild effects in the bacteriocytes (Figs. 3 and 6). These data support the hypothesis that dsRNA administration by microinjection in aphids provokes a much more efficient spread to all the tissues. Therefore, injection into the aphid hemolymph should give access to the different compartments of the aphid body, as has been observed for other insect species (Araujo et al., 2006; Rajagopal et al., 2002; Walshe et al., 2009). In aphids, the absence of an efficient systemic spread of the RNAi effects, following oral administration, may be due to the presence, in the aphid's gut, of a modified perimicrovillar membrane (MPM) (Cristofoletti et al., 2003), equivalent to the peritrophic membrane in other insects. This could act as a natural barrier preventing dsRNA transfer outside the gut. A possible role of the insect gut barrier in preventing the systemic spread of dsRNA has already been reported for the aminopeptidase gene knockdown in *Spodoptera litura* (Rajagopal et al., 2002). Our study also shows that, in aphids, the RNAi effects in the gut and the bacteriocytes are much more important, in terms of the number of individuals affected (for both tissues) and induced phenotypes (for the gut), when dsRNA is administered by feeding. Thus, this administration route remains essential for developing RNAi-based pest control strategies, in the field, against aphids.

The importance of different body compartment targeting related to the dsRNA delivery strategy in aphids was confirmed by the observation of specific phenotypes. Injection of dsRNAs targeting *cathepsin-L* induced a significant modification of the aphid body morphology (20% of treated aphids) and behavior alterations (around 50% of treated aphids). There is a high probability that the alteration of aphid body morphology was related to the inhibition of *cathepsin-L* gene expression in the aphid carcass since *cathepsin-L* expression knockdown was observed in all these aphids. This phenotype defect only appeared in the first 48 h of the experiment, *i.e.* when aphids were around the developmental switch from the 3rd to the 4th instar molting phase. The affected aphids always died 24 h after the observation of the phenotype. This altered body morphology phenotype was not observed by Jaubert-Possamai et al. (2007), who have already performed *cathepsin-L* gene inactivation in the pea aphid using the injection method. A possible explanation is that these authors did not use an individual aphid analysis-based approach following the *cathepsin-L* dsRNA injection. Moreover, the high mortality observed by these authors in the control groups (29% and 38% for water and the control gene respectively, vs. 45% mortality in the *cathespin-L* injected groups) could also have masked the specific effects caused by the target gene inactivation, when compared with the general effects of the trauma induced by microinjection. The localization of *cathepsin-L* mRNA in insects' carcass (Fig. S1, Supplementary data), and the

phenotypes observed following its inhibition (induction of nymph mortality correlated with alteration of aphid body shape) led us to hypothesize that cathepsin-L protein could be involved in the molting process in aphids. Therefore, its inhibition could alter the correct development and, thus, the survival of the aphids. The involvement of cathepsin-L in molting has been demonstrated in nematodes where, either the treatment with enzyme inhibitors (Richer et al., 1993; Lustigman et al., 1996), or RNAi experiments (Hashmi et al., 2002) showed that this cysteine protease is needed for successful molting. In insects, evidence of the crucial role of cathepsin-L in metamorphosis was shown in a holometabolous insect, the cotton bollworm *Helicoverpa armigera,* where this protein is suggested to be involved in the drastic larval tissues destruction necessary for the complete transformation that the larvae undergo to form an adult (Liu et al., 2006; Zhang et al., 2013). Our work shows, for the first time, a possible involvement of cathepsin-L in molting in a hemimetabolous insect. Even if insects belonging to this group go through gradual changes as they turn into adults, as the insect grows, it sheds its cuticle, which cannot grow. Therefore, at each nymph phase transition, a new larger one replaces the old smaller cuticle. Cathepsin-L, and other cysteine proteases could be involved in the extensive degradation of proteins taking place during cuticle remolding in molting of hemimetabolous insects, and this function requires further investigation.

Interestingly, 120 h after the administration of dsRNA, the levels of *cathepsin-L* mRNA were still strongly inhibited in the carcass, but no morphological defects were observed in the treated aphids. It is important to note that, 120 h after the injection, aphids in our treatment groups had already completed their molting from the 4th instar stage to the adult life phase, as this process is normally occurring 96 h after the moment we chose for dsRNA administration. This could be due to a different role or involvement of the *cathepsin-L* gene in aphid molting according to the different nymph stages switches.

On the other hand, the administration of *cathepsin-L* dsRNA by feeding induced a high mortality rate and an alteration of the gut morphology in treated aphids, when compared to the control groups. Inhibition of gut cysteine proteases by the use of enzymatic inhibitors is known to cause external morphological defects during development, arrested growth and mortality in insects (Stotz et al., 1999; Murdock and Shade, 2002; Tamhane et al., 2007; Parde et al. 2010). Although weakly, aphid mortality had already also been observed following cysteine proteases inhibition by an oral route (Cristofoletti et al., 2003; Azzouz et al., 2005). Nevertheless, our RNAi approach specifically targeting *cathepsin-L* gene was more efficient in killing aphids. Depletion of intestinal proteases is considered able to induce (and deregulate) the synthesis of other proteases, which could result in toxic effects altering insect development. This study is the first to report that the specific inhibition by RNAi of a cysteine protease causes an alteration of gut cells during insect development. Indeed, a further detailed histological analysis allowed us to observe number of lyzed cells and necrotic vacuoles in the anterior midgut of aphids appearing after feeding on *cathepsin-L* dsRNA-supplemented diet. This phenotype is probably caused by the specific inhibition of *cathepsin-L* gene in the aphid gut following the feeding treatment. In fact, the highest mortality rate for the feeding treatment and the peak in the deterioration of aphid stomach cells both took place 120 h after the start of dsRNA administration, therefore following the most significant *cathepsin-L* mRNA knockdown in the aphid's gut (observed 72 h after the beginning of the treatment). Previous studies on cathepsin-L localization have shown that this protein is found mostly in the anterior part of the gut and, more specifically, in vesicular and modified perimicrovillar structures (Deraison et al., 2004), but its sub-cellular localization has never been clearly shown. On the basis of our results, our aim was to provide more precise information on cathepsin-L expression in aphid gut cells. To achieve this, we used a new antibody recently developed against insect cathepsin-L, which is more specific than that previously employed. With this approach, we found a prominent and clear localization of cathepsin-L in the stomach cells (Fig. 7), proving that this protein is expressed in the same cells of the aphid gut as those where we observed the biggest and specific tissue deterioration following the gene knockdown caused by RNAi.

More generally on the aphid reaction to RNAi treatment, we were able to detect that variation in the knockdown effects exists among different individuals even when they have received the same treatment. This observation that, regardless of the administration method used, the RNAi will not have a uniform effect on all the individual aphids, has been previously shown for other insect species (Amdam et al., 2003; Dong and Friedrich, 2005; Marshall et al., 2009). In aphids, a difference in the response to RNAi treatment between different pools was already observed in the previous microinjection study performed by Jaubert-Possamai et al. (2007). This variation could be explained by the physiological condition of each individual insect at the time of treatment, which can also have a consequence on the target gene expression knockdown. It is worth noting that, for the injection experiments, the dsRNA quantity administered to aphids is easily controlled, whilst, in the feeding experiments, individual variability may also be due to differences in dsRNA intake. Therefore, the individual feeding

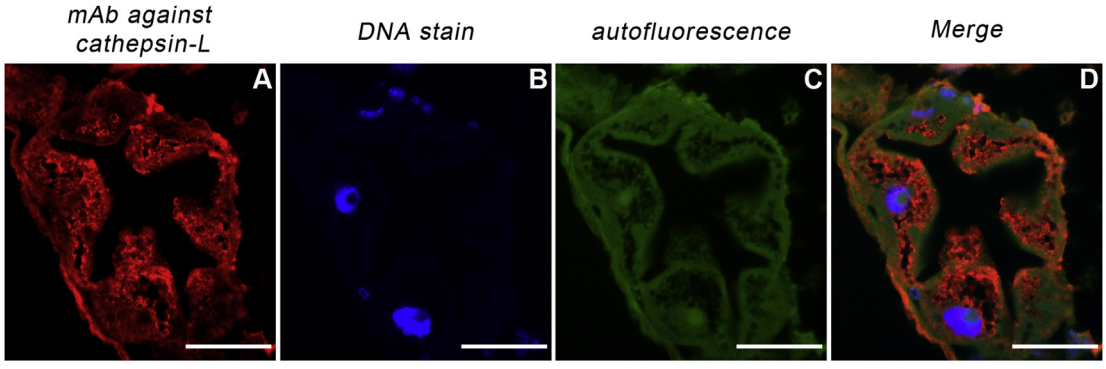

Fig. 7. Localization of cathepsin-L protein in the pea aphid gut. (A) Sections of *A. pisum* gut stained with the anti-cathepsin-L mouse mAb, using the goat anti-mouse Alexa-Fluor568 as a secondary antibody. (B) Nuclear staining with DAPI. (C) Autofluorescence corresponding to non-specific fluorescence naturally emitted by aphid tissues, as photographed using a FITC filter set (green signal). (D) Merged figure. The images represent a single optical section observed under an epi-fluorescence microscope (Olympus IX81). Scale bars are 20 μm.

behavior of any single aphid could, in part, explain the high individual variability of gene knockdown observed in our feeding experiments. Finally, dsRNA appears to be susceptible to degradation by aphid salivary secretions and by aphid hemolymph (Christiaens et al., 2014), which could also contribute to variable effect of the RNAi treatment on individual aphids.

Other important factors for the success of RNAi experiments are the duration and the timing of gene expression knockdown, because both of these parameters potentially affect the expression of detectable phenotypes. In our study, the peak in *cathepsin-L* inhibition was observed from 72 h after the beginning of the treatment. These results are consistent with most of those from previous RNAi studies in aphids (Jaubert-Possamai et al., 2007; Mutti et al., 2006; Whyard et al., 2009; Guo et al., 2014). Only Shakesby et al. (2009) observed the strongest RNAi knockdown effect 24 h after the start of the feeding treatment in aphids. This difference (24 *vs.* 72 h for the highest knockdown effect) could be an example of how the combination of the expression pattern and the administration method used is leading to the temporal pattern of gene expression down-regulation of a given target gene.

To summarize, our study highlights the importance of individual and tissue specific analyses to characterize precisely the phenotypic response to RNAi treatment in insects. We optimized two administration methods (injection and ingestion) and we have shown that the dsRNA administration alone is able to induce lethal effects above certain dose thresholds in aphids. Our individual analysis of treated aphids enabled us to observe phenotypes never previously seen in these insects following RNAi treatment, and to show a clear relationship between *cathepsin-L* gene knockdown and body compartment-specific reactions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2014.05.005.

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